Antonie van Leeuwenhoek **78:** 297–305, 2000.

Streptomycetes in micro-cultures: Growth, production of secondary metabolites, and storage and retrieval in the 96-well format

Wolfgang Minas*, James E. Bailey & Wouter Duetz
*Institute of Biotechnology, ETH-Zürich, 8093 Zürich, Switzerland (*Author for correspondence; E-mail: minas@biotech.biol.ethz.ch)*

Received 10 April 2000; accepted 19 July 2000

Key words: actinorhodin, microbial cultivation, secondary metabolites, *Streptomyces arenae*, *Streptomyces coelicolor*

Abstract

Mycelium-forming *Streptomyces* strains were grown in one milliliter liquid micro-cultures in square deep-well microtiter plates. Growth was evaluated with respect to biomass formation and production of secondary metabolites which were found to be very similar in the micro-cultures, bioreactor, and shake flask cultivations, respectively. Despite repetitive sampling and extensive growth on the walls of the wells, no cross contamination occurred. Furthermore, we successfully employed cold storage at −20 °C of spore suspensions (in the 96-well format), directly prepared from cultures grown on agar in the microtitre plate. Cultures were retrieved by replicating aliquots from the frozen spore suspensions.

Abbreviations: act – actinorhodin biosynthetic genes; ARO – aromatase; CDW – cell dry weight; CYC – cyclase; DMAC – 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid; KR – ketoreductase; MOPS – 3[N-morpholino]propanesulfonic acid; MTP – microtiter plate; PKS – polyketide synthase; rpm – revolutions per minute; *rub* – rubromycin biosynthetic gene cluster; v/v – volume by volume; vvm – volume by volume by minute

Introduction

Screening applications such as media optimizations, search for new bioactive molecules or characterization of recombinant/mutagenized organisms, require examination of large numbers of cultures. In this respect it is advantageous when representative and reproducible cultivations can be done in small scale, preferably in a standard format that is amenable to automation. A recently described cultivation system (Duetz et al. 2000) makes use of the 96-square-deep-well microtiter plate (MTP) format. Oxygen transfer rates in this system were as high as 38 mm O_2 l^{-1} h^{-1} using 0.5 ml volume or 18 mmol O_2 l^{-1} h^{-1} in 1 ml culture volume. Approximately 9 g/l biomass were obtained with *Pseudomonas putida* growing on glucose minimal medium. Other aerobic *Pseudomonas* and *Rhodococcus* strains were also evaluated. In addition, a replication and storage scheme was developed that allowed for long term cold storage and retrieval of strain collections as stock cultures in the 96 well format.

We are interested in secondary metabolism of streptomycetes which involves screening recombinant strains of *Streptomyces* for the production of novel secondary metabolites, comparing media best suited for sporulation or secondary metabolite production, and physiological studies on factors that affect morphological and physiological differentiation. Generally, mycelial organisms such as streptomycetes are difficult to grow in small scale and the reproducible production of secondary metabolites has to be evaluated in several parallel cultures.

Here we report on growth and secondary metabolite formation by several *Streptomyces* strains in 1 ml liquid cultures using square deep-well plates, and on how these cultures compare to larger scale shake flask or bioreactor cultivations. We also show that the

Table 1. Strains and plasmids used in this study.

Strain/Plasmid	Relevant phenotype	Reference
S. coelicolor A3(2)	Wild-type, SCP1, SCP2	Hopwood et al. 1985
CH999	*S. coelicolor* A3(2) derivate Δ*act*, *redE60*, *argA*1/*pro*A1, no growth in liquid on maltose	Khosla et al. 1992; McDaniel et al. 1993
S. lividans TK24	Actinorhodin producer	Hopwood et al. 1985
S. arenae DSM 40737	Wild-type, naphthocyclinones	Pridham et al. 1958
S. collinus DSM 2012	Wild-type rubromycins	Lindenbein 1952
WM2303	CH999(pIJ2303), actinorhodin	this work
RM5	CH999(pRM5), DMAC, AleosaponarinII	McDaniel et al. 1993
pIJ2303	SCP2*, *act*	Malpartida & Hopwood 1984
pRM5	SCP2*, *act* KR, PKS, ARO, CYC	McDaniel et al. 1993

act: actinorhodin biosynthesis genes from *S. coelicolor* A3(2).
rub: putative rubromycin biosynthesis genes from *S. collinus* DSM2012.

system is suitable for preparation of spore stocks for maintaining a *Stretptomyces* strain collection.

Material and Methods

Strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *S. coelicolor* CH999 was transformed using the PEG-mediated transformation protocol described in Hopwood et al. (1985) with plasmids pRM5 (McDaniel et al. 1993) and pIJ2303 (Malpartida & Hopwood 1984), yielding strain RM5 making aleosaponarin II and 3,8-dihydroxy-1-methyl-anthraquinone-2-carboxylic acid (DMAC) and strain WM2303 producing actinorhodin (Table 1). Both plasmids confer thiostrepton resistance and allow developmentally controlled expression of the biosynthetic genes for the respective products.

For spore preparations, the *S. coelicolor* strains and the *S. arenae* strain were grown at 30 °C on solid R2YE medium (Hopwood et al. 1985). Frozen spore suspensions ($\sim 10^6$ ml^{-1}) were prepared as described elsewhere (Hopwood et al. 1985). *S. collinus* did not sporulate on R2YE or on other commonly used media. Therefore, frozen mycelium was used as inoculum. Liquid cultures of all strains were grown at 30 °C in TSB (Oxoid), YEME (Hopwood et al. 1985), or a modified MG medium (Doull & Vining 1989). Briefly, one liter MG medium was prepared by dissolving in 780 ml water L-glutamate monosodium (60 mM, Fluka), $MgSO_4$ * 7 H_2O (0.2 g, Merck), $FeSO_4$ * 7 H_2O (9 mg, Merck), $CaCl_2$ (1 mg, Merck), NaCl (1 mg, Merck), MOPS (21 g, Fluka), adding 15 ml 1 M potassium phosphate solution pH 6.5 and 4.5 ml trace element solution (containing per liter 39 mg $CuSO_4$ * 5 H_2O (SIGMA), 5.7 mg boric acid (Merck), 3.7 mg $(NH_4)_6Mo_7O_{24}$ * 4 H_2O (SIGMA), 6.1 mg $MnSO_4$ * 1 H_2O (SIGMA), and 880 mg $ZnSO_4$ * 7 H_2O (SIGMA). Maltose, 50 g (Fluka), dissolved in 200 ml water, was sterilized separately and added aseptically to the autoclaved salt solution. Since some strains required arginine and proline, MG medium was always supplemented with 37 mg l^{-1} (final concentration) arginine and 1 g l^{-1} proline. Where needed, thiostrepton was added to 50 mg l^{-1} (final concentration). MG used in bioreactor cultivations was without MOPS.

Unless stated otherwise, liquid media cultivations were performed in two stages: a spore germination and the actual cultivation. All shake flask cultivations were done in standard erlenmeyer flasks containing a stainless steel spring (∼10 mm diameter) coiled on the bottom of the flask for better aeration. For the spore germination stage, flasks filled with medium to 1/5th of the total volume were inoculated with 1/1000 (v/v) of the spore suspension and grown at 30 °C with 300 rpm on a gyratory shaker incubator (G-25, New Brunswick Scientific). This preculture was used after 48 hours incubation (72 h were needed for the slow growing *S. collinus* and RM5 strains). The timing was such that a dense preculture was obtained that had not yet started secondary metabolism, indicated by the color of the broth. Bioreactors were inoculated with 5% (v/v) preculture and operated for up to 4 weeks (only the first 15 days are relevant for this study). Cultivations were performed in LH 210 bioreactors (Inceltech LH SGI S.A., France) with 4 liter working volumes under ambient pressure, sparged with sterile air (0.25 vvm), and agitated at 700 rpm using a pitched blade

turbine to counteract rapid settling of mycelial aggregates. The temperature was set to 30 °C and the pH maintained at 6.5 using 0.5 M sulfuric acid and 0.5 M sodium hydroxide, respectively. The process was controlled by the Incelsoft software, with on-line data collected at regular intervals for pH, dissolved oxygen, biomass (optical biomass sensor by Wedgewood, as long as cells remained dispersed in the broth), and for CO_2 and O_2 concentrations from mass spectroscopic off-gas analysis (VG Prima 600, VG Gas Analysis Systems, Middlewich, UK). Samples (15 ml) for off-line measurements were withdrawn aseptically and chilled on ice. Two ml of the samples were filtered through a 0.2 μm syringe filter for use in the biochemical analysis. All samples were stored at −20 °C and analyzed together at the end of the cultivation.

Shake flask cultivations were done in 200 ml MG inoculated with 1% preculture and incubated at 30 °C with 300 rpm on a gyratory shaker incubator (G-25, New Brunswick Scientific).

Liquid micro-cultures in a 1 ml scale (inoculated with 1 μl spore suspension or 10 μl preculture) were done in polypropylene deep well plates with 40 mm deep wells with a cross-section of 8.2×8.2 mm (Megaplate, Polylabo, Geneva, Switzerland). Plates were mounted in a special holder (Kühner AG, Basel, Switzerland) and incubated at 30 °C with shaking at 300 rpm in a gyratory shaker (5 cm displacement). Oxygen transfer rates of 18 mmol O_2 l^{-1} h^{-1} were previously determined for this setup (Duetz et al. 2000). A second type of deep-well plate (Bell-Art, USA) with thicker walls and a polypropylene lid was used for storage of culture stocks at −80 °C. Prior to use, all plates were treated with acid and base to remove toxic UV-absorbers, antioxidants, whiteners, and heavy metals, as described by Duetz and coworker (Duetz et al. 2000).

Biochemical assays and biomass determination

Actinorhodin concentrations were determined spectrophotometrically as described previously (Bystrykh et al. 1996). Briefly, samples (50 μl) from micro-cultures were transferred into flat-bottom 96-well MTPs, an equal volume 2 M KOH was added before a spectrum from 400–700 nm of each well was recorded with a SPECTRAmax Plus MTP reader (Molecular Devices, USA). The actinorhodin concentration was calculated using the molar extinction coefficient ϵ_{640}= 25,320, a molecular mass of 634.65 assuming a 3 mm path length in the wells. Larger samples were measured in standard 1 cm path cuevettes. Cell dry weight (CDW) in micro-culture cultivations was determined by collecting the broth and the biomass attached to the walls. After determination of the total culture volume, the entire sample was filtered though a preweighted glassfiber filter (GF/A, Whatman). The filters were washed with 2 volumes of water and dried at 80 °C. CDW in shake flask cultivations was determined similarly by withdrawing 10 ml samples from the broth after biomass attached to the wall had been resuspended, followed by the filtration, washing and drying steps. CDW could not be determined in all samples of the bioreactor cultivations because at later stages in the cultivation extensive growth on the walls and in the headspace interferred with representative sampling. In this case, biomass concentration was determined from the nitrogen consumption based on the general assumption that nitrogen is not mineralized and that a *Streptomyces* cell also contain 14% nitrogen (similar to other bacteria). Arginine, glutamate and proline were the sources of nitrogen in the medium. Concentrations of glutamate and proline were measured in the cleared culture supernatant by analytical HPLC using a C8 reverse phase column (Nucleosil 100-5-C8 5 μm 250×4.6, Bischoff, Leonberg, GER) and 0.2 N H_2PO_4 as mobile phase (0.7 ml/min) and detection at 210 nm. Free ammonium concentrations in the filtered broth were determined using the Dr. Lange LCK303 cuvette test in combination with the Dr. Lange CADAS 30 Photometer (Dr. Lange AG, Hegnau, Switzerland). The amount of nitrogen used for biomass production is calculated from the difference between the nitrogen initially supplied in the medium (arginine, glutamate and proline) and the recovered nitrogen in the broth (remaining glutamate, proline and released ammonium). Arginine concentrations, accounting for <10% of the total nitrogen in the broth, could not be resolved by HPLC. For the calculations it is assumed to be assimilated and incorporated into the biomass.

Results

Growth in 1 ml micro-cultures

Growth of *S. coelicolor* A3(2), WM2303, RM5 and *S. lividans* TK24 in 1 ml micro-cultures was compared to growth in larger scale shake flask or bioreactor cultivations. Twelve wells of a 96-square-deep-well plate were each filled with 1 ml of spore suspensions in MG medium. The medium used for strains

Table 2. Biomass and secondary metabolite concentrations obtained from 1 ml micro-cultures on MG medium after 15 days incubation. The data represent values obtained after pooling 10 representative cultivations

Strain	Volume recovered ml	Volume lost %	CDW g/l	Actinorhodin mg/l	RM5[1] OD_{450}
S. coelicolor A3(2)	9.6	4	8.1	113	
WM2303	9.35	6.5	6.3	154	
RM5	9.6	4	1.6		1.5
S lividans TK24	9.25	7.5	9.5	63	

[1]RM5 produces a mixture of Aleosaponarin II and DMAC which have an absorption maximum of 450 nm. Since no molar extinction coefficients are available the raw OD data are presented.

Table 3. Final actinorhodin concentrations in cleared culture supernatant of MG grown cultures after up to 15 days' incubation at 30 °C. Culture volumes were 1 ml, 200 ml and 4 l for micro-cultures, shake flasks and bioreactor cultivations, respectively

	Actinorhodin (mg/l)					
	Micro-cultures		Shake flask		Bioreactor	
Days of incubation:	5	15	5	6	5	15
S. coelicolor A3(2)	8	115±2.8[a]				
S. coelicolor A3(2)				117		
WM2303		154[b]				
WM2303	14±0.4[c]	163±9.1[c]				
WM2303				114±26.8[d]		
WM2303					62±10.6[e]	214
S. lividans TK24		63[b]				
S. lividans TK24			61±16.7[f]			

[a] Averages from two experiments with twelve replicates each.
[b] Data from growth experiment described in Table 2.
[c] Averages from four experiments with six replicates each.
[d] n=4 shake flask cultures.
[e] n=2 bioreactor cultivations.
[f] n=3 shake flask cultures.

WM2303 and RM5 contained thiostrepton. After 15 days of cultivation, broth and biomass was recovered from 10 representative wells of each strain. The total recovered culture volume was recorded and the cleared supernatant used for quantification of secondary metabolite concentrations. The biomass was used for CDW determination. The results, the average of the ten wells, are summarized in Table 2. Since cultivations of streptomycetes are conducted over extended periods of time, it was important to see that after 15 days of cultivation the loss of culture volume due to evaporation was minimal (4 to 7.5%). In the case of *S. coelicolor* A3(2), 8.1 g/l CDW in micro-cultures compared well with 7 g/l biomass reached in shake flask cultivations after 7 days, after carbon and nitrogen sources were depleted. The best growth was observed for *S. lividans* TK24 which yielded 9.5 g/l in micro-cultures. Strain WM2303, grown in micro-cultures, resulted in a significantly higher biomass, 6.3 g/l CDW compared to only 3.5 to 4 g/l biomass obtained in shake flask (7 days) or bioreactor cultivations (15 days). In contrast, RM5, a generally slower growing strain, yielded only 1.5 g/l CDW in micro-cultures while approximately 2.5 g/l were obtained in larger scale cultivations (15 days). The final concentrations of secondary metabolites were also determined (Table 2) these compared well with the amounts obtained from a second set of micro-cultures and shake flask or bioreactor cultivations (see below, Table 3).

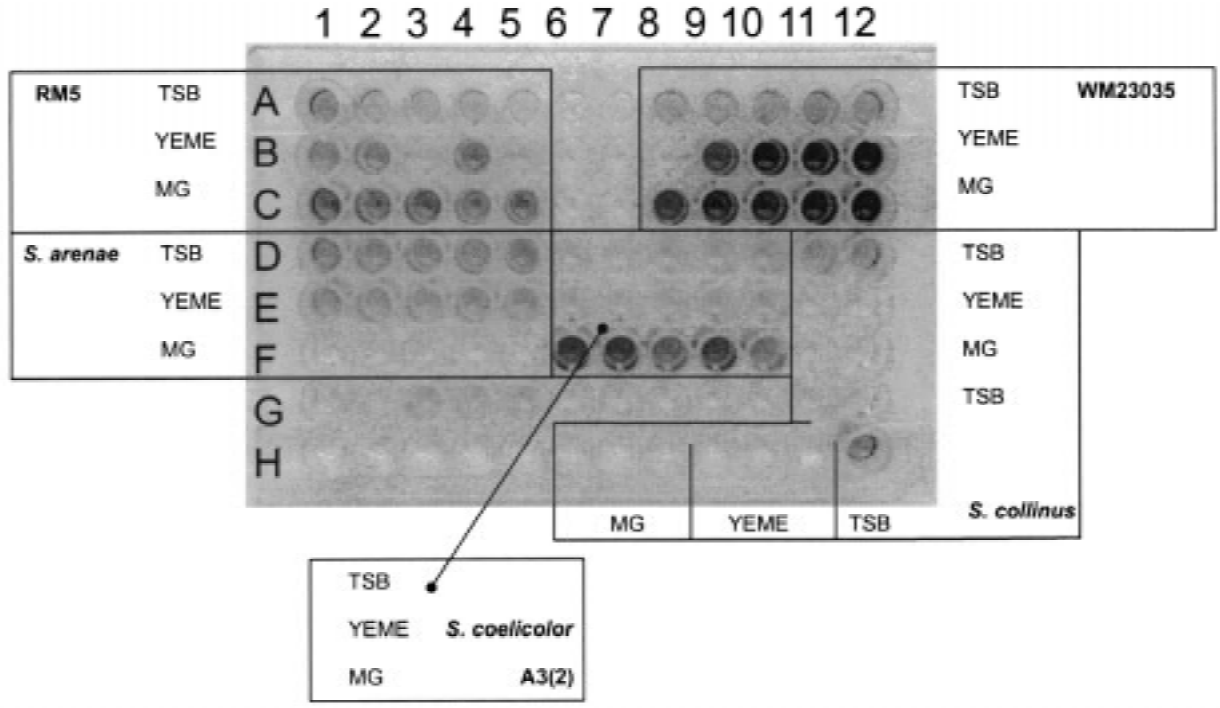

Figure 1. Growth and production of secondary metabolites by streptomycetes grown on different media in micro-cultures. The microtiter plate shown contains 50 μl samples taken from a deep-well plate after 15 days incubation. Growth and production of secondary metabolites was observed with the *S. coelicolor* A3(2) (D6–F10), *S. arenae* (D1–F5), and the recombinant strains RM5 (A1–C5) and WM2303 (A8–C12). *S. collinus* did not grow (see text). A6–C7 were sterility controls (i.e. wells that contained sterile medium only).

Production of secondary metabolites

The time course of secondary metabolite production was evaluated on 3 different media with 5 *Streptomyces* strains. The media were inoculated from spore suspensions, with the exception of *S. collinus* where frozen mycelium was used. One ml aliquots of each culture were dispensed into 5 wells of a 96 square deep-well plate (Layout see Figure 1) and placed on a shaker. Samples (50 μl) were withdrawn aseptically and transferred into a flat bottom MTP for spectrophotometric analysis. First the absorbance between 400 and 700 nm was determined for each well, then the 2 M KOH was added for actinorhodin determination and the plate was measured again (Figure 1). For cultures that make secondary metabolites other than actinorhodin, the raw absorption data from the first measurement are presented since molar extinction coefficients were not available.

Similar to other forms of liquid cultivation, heavy growth occurred on the walls above the liquid phase. Biomass which remained in the broth formed pellets 1–3 mm in diameter. Pellet formation was less pronounced in MG medium compared to TSB or YEME. Despite repeated withdrawals of samples, no cross-contaminations were observed and all of the control wells remained sterile.

The results show that the maximum growth rates and secondary metabolite production were different for the various strains. While all *S. coelicolor* derivates preferred MG medium and to a lesser extent YEME medium, *S. arenae* showed best production of secondary metabolites on TSB. *S. collinus* did not grow well in liquid culture, this was attributed to the use of frozen mycelium as inoculum (*S. collinus* did not sporulate on any commonly used medium) which is more difficult to aliquot and which has a lower viability than spores. It was notable to see that the time course as well as the amounts of actinorhodin produced by *S. coelicolor* A3(2), *S. lividans* TK24, and WM2303 were similar to those determined in the larger scale shake flask or bioreactor cultivation. (Table 3 and Figure 2A–D). Production of actinorhodin by *S. coelicolor* A3(2) in micro-cultures and in shake flasks leveled off at about 115 mg/l after 150 to 200 h cultivation (Figure 2A, C). In contrast, strain WM2303 continues to produce actinorhodin through to the end of

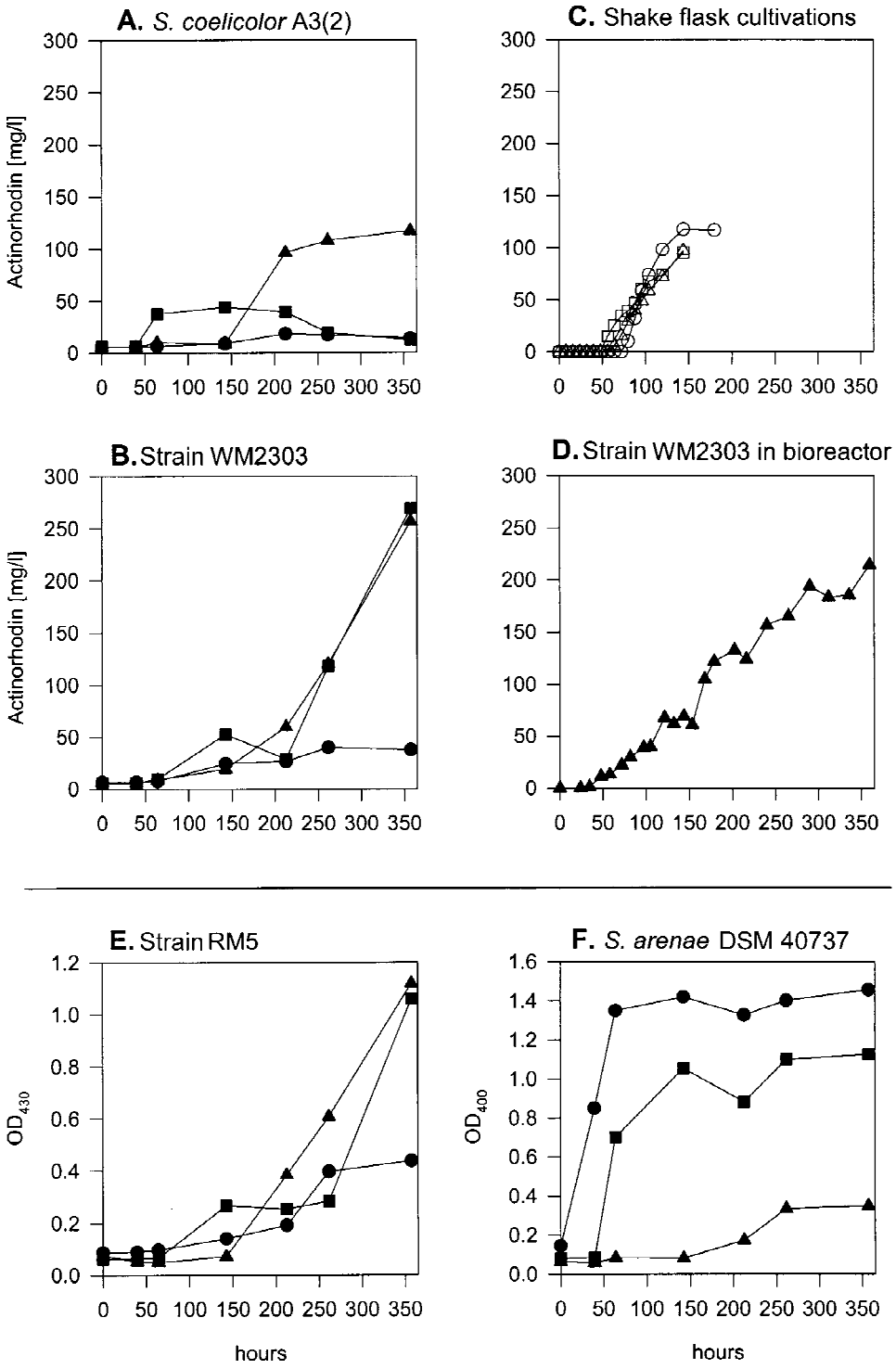

Figure 2. Time course of secondary metabolite production by different strains of *Streptomyces* during 15 days', cultivation in 1 ml micro-cultures, shake flask or bioreactor cultivation. The 4 actinorhodin producing cultures are grouped on top (panel A–D) for better comparison. Filled triangles indicate MG grown cultures, filled squares YEME and filled circles TSB grown cultures. Open symbols (panel C) represent shake flask cultivations done in MG (open circle: *S. coelicolor* A3(2); open square and triangle: strains WM2303). Data from micro-cultivations represent mean values of 3 to 5 parallel cultivations (wells). Raw absorption data are presented for the RM5 and *S. arenae* strains. The maximum absorption of their secondary metabolites were at 400 and 430 nm, respectively.

Table 4. Maximum growth rates determined in micro-cultures, shake flasks and bioreactor cultivations. Growth in micro-cultures and bioreactor cultivations was measured by optical density of the broth at 600 nm (cells grow well dispersed during early exponential phase) measurements. In shake flask cultivations, cell dry weight was determined

Strain	Medium	Cultivation temperature	μ_{max}	Number of replicates	Standard deviation
Micro-cultures					
WM2303	MG	30 °C	0.0545	24 (4×6)[d]	0.0041
WM2303	MG	25 °C	0.0284	5	0.0053
WM2303	TSB	25 °C	0.0158	5	0.0118
WM2303	YEME	25 °C	0.0199	4	0.0050
S. coelicolor A3(2)	MG	25 °C	0.0607	4	0.0297
S. coelicolor A3(2)	TSB	25 °C	0.0050	5	0.0027
S. coelicolor A3(2)	YEME	25 °C	0.0309	5	0.0029
RM5	MG	25 °C	0.0123	5	0.0039
RM5	TSB	25 °C	0.0029	5	0.0011
RM5	YEME	25 °C	no growth	5	–
S. arenae	MG	25 °C	no growth	5	–
S. arenae	TSB	25 °C	0.0601	5	0.0022
S. arenae[c]	YEME	25 °C	0.0137	5	0.0126
Shake flasks[a]					
WM2303	MG	30 °C	0.1062	1	–
S. coelicolor A3(2)	MG	30 °C	0.1049	1	–
CH999	MG	30 °C	0.1085	1	–
Bioreactor cultivations[b]					
WM2303	MG	30 °C	0.1084	2	0.0039

[a] 200 ml volume.
[b] 4 liter volume.
[c] Weak growth only.
[d] The averages from four experiments with six replicates each were used to determine the standard deviation.

the cultivations (Figure 2B–D) yielding 260 mg/l actinorhodin. RM5 (Figure 2E) behaved similar to strain WM2303 even though RM5 makes aleosaponarin II and DMAC. *S. arenae*, which is not *S. coelicolor* derived, showed a different pattern of growth and secondary metabolite production. This strain produced secondary metabolites (naphthocyclinones, Brünker et al. 1999) when grown on TSB which also supported faster growth than the minimal medium MG (Table 4).

Except for an extended lag phase in the micro-cultures, which required cultures to be continued for 2 weeks as compared to only one week for shake flask cultivations, secondary metabolite production was similar between micro-cultures and larger scale cultivations (Table 3). Culture-to-culture variations were found to be smallest in mirco-cultures. Maximum growth rates in shake flask and bioreactor cultivations were about 0.1 h^{-1}, while deep-well cultures grew at about half this rate (Table 4).

Storage and retrieval

Storage of a strain collection in a MTP-format is straightforward for e.g. genomic libraries in *E. coli*. The suitability of the micro-culture system for the preparation of frozen stocks of *Streptomyces* spores was tested. The wells of a Bell-Art deep-well plate were filled with 0.3 ml of R2YE agar medium supplemented with the appropriate antibiotics. The wells were inoculated with 0.5 μl from the respective spore stocks. After incubation for 2 weeks at 30 °C all cultures had sporulated. Incubation was extended until the ager had dried completely. Then 100 μl amounts of a sterile 15% glycerol solution were directly added to each well and spores resuspended by scraping the dried agar and mixing with a sterile 96-well replicator device (Duetz et al., 2000). Spore suspensions could now be frozen and stored at −20 °C.

Analogous to the technique described for *Pseudo-*

monas or *Rhodococcus* (Duetz et al. 2000), retrieval of *Streptomyces* from frozen stocks involved 2 steps. First, a sterile replicator (initially at room temperature) is pressed onto the frozen surface for three seconds. The heat transfer from the pins melts approximately 0.3 μl of the frozen stocks. This liquid forms a thin layer on the pin's surface which is then transferred onto an agar plate. This plate is incubated until growth of the replicated cultures is visible. Liquid cultures can subsequently be inoculated from this master plate by using the 96-well replicator. This approach worked well with triplicates of the 7 *Streptomyces* strains including *S. coelicolor* A3(2), WM2303, RM5, *S. arenae* DSM 2010, *S. collinus* DSM 40737, and 2 additional recombinant *S. coelicolor* CH999 derived strains. All cultures grew, sporulated in the deep well from plates yielding frozen stocks from which viable cultures could be recovered (data not shown).

Discussion and conclusions

A new microtiter-based cultivation system has been tested for use with streptomycetes. The system had been optimized in respect to high oxygen transfer rates (Duetz et al. 2000). Oxygen transfer rates of up to 38 mmol O_2 l^{-1} h^{-1} could be obtained which is equivalent to what is obtained in a 250 ml Erlenmeyer flask filled with 25 ml medium under identical shaking conditions.

The dissolved oxygen tension in the broth (Yegneswaran & Gray, 1991; Clark et al. 1995; Okabe et al. 1992), the medium, namely the sources of carbon and/or nitrogen and phosphate (Hobbs et al. 1990, Liao et al. 1994; Melzoch et al. 1997), but also the size and type of inoculum or type of vessel and agitation will all greatly affect growth of *Streptomyces* strains in liquid culture and the production of secondary metabolites (Whitacker 1992). In this respect it was notable that the *Streptomyces* strains tested did grow very well in the square deep-well plates. Only *S. collinus* did not grow, this, however, was attributed to the type of inoculum used. Biomass yields were similar or better than in larger scale shake flask or bioreactor cultivations. Only strain RM5 grew less well in the micro-cultures as compared to bioreactor cultivations. The increased biomass yield in the micro-cultures could be related to the heavy growth covering the walls of the wells above the liquid phase, this, however, is also observed in bioreactor cultvations after 6 days (Minas, unpublished observation). The attached growing mycelium appears to grow faster than the submerged pellets as mycelial microorganisms are adapted to grow on solid substrates (Prosser & Tough 1991).

In addition to the higher surface to volume ratio in the square wells compared to shake flasks or bioreactors, the material of the deep-well plates (polypropylene) provided a good substrate for attachment, growth and morphologically differentiation. Aerial hyphae formation and sporulation was observed for non-submerged parts of the cultures. Since morphological differentiation has far-reaching consequences for the metabolism of the cells, it could also explain the high biomass obtained from strain WM2303 grown on MG. This strain is deficient for growth on maltose when grown in submerged culture. Thus, it can only utilize glutamate and proline as sources of carbon and energy resulting in 3.5 to 4 g/l CDW in shake flask and bioreactor cultivations. This compares to 6.3 g/l determined in the micro-cultures. The possibility that maltose deficiency is overcome during morphological differentiation (which remains to be tested) could explain the better growth in the deep-wells. Differences were observed in the lag phase and the maximun growth rates determined for the micro-cultures. An extended lag phase was observed in the 1 ml-cultures and maximum growth rates were about half that of larger scale cultures.

Secondary metabolite levels were within the range of those determined in shake flask or in bioreactor cultivation. Comparison between different media showed that the *S. coelicolor* derived strains preferred MG or YEME as media for secondary metabolite production while *S. arenae* performed best on TSB medium. Similar to shake flask cultivations, well-to-well and plate-to-plate variations in secondary metabolite production were observed, this, however, was smallest in micro-cultures. Studies of a large number of replicate micro-cultivations can easily be performed which allows one to discriminate between differences due to culture-to-culture variations and real effect of, e.g., a physiological condition. The system was also successfully tested for cold storage of streptomycete stock cultures.

In conclusion, this new micro-cultivation technology facilitates cost- and labour-efficient, massive parallel cultivations of streptomycetes for screening purposes or for physiological studies. We are currently using this technology to evaluate physiological parameters on growth and secondary metabolite biosynthesis and in bioconversion assays with our recombinant polyketide synthesizing strains.

Acknowledgements

WM was supported by a Junior Group Leader program of the Swiss National Science Foundation Grant Nr 5002-46086.

References

Brünker P, McKinney K, Sterner O, Minas W & Bailey JE (1999) Isolation and characterization of the naphthocyclinone gene cluster from *Streptomyces arenae* DSM 40737 and heterologous expression of the polyketide synthase genes. Gene 227: 125–135

Bystrykh LV, Fernández-Moreno MA, Herrema JK, Malpartida F, Hopwood DA & Dijkhuizen L (1996) Production of actinorhodin-related 'blue pigments' by *Streptomyces coelicolor* A3(2). J. Bacteriol. 178: 2238–2244

Clark GJ, Langeley D & Bushell ME (1995) Oxygen limitation can induce microbial secondary metabolite formation: investigations with miniature electrodes in shaker and bioreactor culture. Microbiol. 141: 663–669

Doull JL & Vining LC (1989) Culture conditions promoting dispersed growth and biphasic production of actinorhodin in shaken cultures of *Streptomyces coelicolor* A3(2). FEBS Microbiol. Lett. 65: 265–268

Duetz W, Rüedi L, Hermann R, O'Conner K, Büchs J & Witholt B (2000) Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Appl. Environ. Microbiol. 66: 2641–2646

Hobbs G, Frazer CM, Gardner DCJ, Flett F & Oliver SG (1990) Pigmented antibiotic production by *Streptomyces coelicolor* A3(2): Kinetics and the influence of nutrients. J. Gen. Microbiol. 136: 2291–2296

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Burton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM & Schrempf H (1985) Genetic manipulation of Streptomyces – A laboratory manual. The John Innes Foundation, Norwich, UK

Khosla C, Ebert-Khosla S & Hopwood DA (1992) Targeted gene replacements in a *Streptomyces* polyketide synthase gene cluster: role for the acyl carrier protein. Mol. Microbiol. 6: 3237–3249

Liao X, Vining LC & Doull JL (1995) Physiological control of trophophase-idiophase separation in streptomycete cultures producing secondary metabolites. Can. J. Microbiol. 41: 309–315

Lindenbein W (1952) Über einige chemisch interessante Aktinomycetenstämme und ihre Klassifizierung. Arch. Mikrobiol. 17: 361–383

Malpartida F & Hopwood DA (1984) Molecular cloning of the whole biosynthetic pathway of a *Streptomyces* antibiotic and its expression in a heterologous host. Nature 309: 462–464

McDaniel R, Ebert-Khosla S, Hopwood DA & Khosla C (1993) Engineered biosynthesis of novel polyketides. Science 262: 11542–11546

Melzoch K, Teixeira de Mattos MJ & Neijssel OM (1997) Production of actinorhodin by *Streptomyces coelicolor* A3(2) grown in chemostat culture. Biotechnol. Bioeng. 54: 577–582

Okabe M, Kuwajima T, Satoh M, Kimura K, Okamura K & Okamoto R (1992) Preferential and high-yield production of a cephamycin C by dissolved oxygen controlled fermentation. J. Ferment. Bioeng. 73: 292–296

Pridham TG, Hesseltine CW & Benedict RG (1958) A guide for the classification of streptomycetes according to selected groups. Appl. Microbiol. 6: 52–79

Prosser JI & Tough AJ (1991) Growth mechanisms and growth kinetics of filamentous microorganisms. Crit. Rev. Biotechnol. 10: 253–274

Whitaker A (1992) Actinomycetes in submerged culture. Appl. Biochem. Biotechnol. 32: 23–35

Yegneswaran PK, Gray MR & Thompson BG (1991) Effect of dissolved oxygen control on growth and antibiotic production in *Streptomyces clavuligerus* fermentations. Biotechnol. Prog. 7: 246–250